Pergamon

Biochemical Pharmacology, Vol. 47, No. 6, pp. 1079–1087, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0050-H

ROLE OF CELLULAR GLUTATHIONE AND GLUTATHIONE *S*-TRANSFERASE IN THE EXPRESSION OF ALKYLATING AGENT CYTOTOXICITY IN HUMAN BREAST CANCER CELLS

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(*Received* 30 *June* 1993; *accepted* 24 *September* 1993)

Abstract—Glutathione (GSH) and glutathione *S*-transferases (GSTs)† play an important role in the protection of cells against toxic effects of many electrophilic drugs and chemicals. Modulation of cellular GSH and/or GST activity levels provides a potentially useful approach to sensitizing tumor cells to electrophilic anti-cancer drugs. In this study, we describe the interactions of four representative alkylating agents (AAs), melphalan, 4-hydroperoxy-cyclophosphamide (4HC), an activated form of cyclophosphamide, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), and cisplatin, with GSH and GST in the human breast cancer cell line MCF-7. Depletion of cellular GSH pools by ~80% by treatment of the cells with the GSH synthesis inhibitor buthionine sulfoximine (BSO) sensitized the tumor cells to each AA to a different extent, with dose-modifying factors of 2.39, 2.21, 1.64, and 1.27 observed for melphalan, 4HC, cisplatin, and BCNU, respectively. Treatment of the cells with the GST inhibitor ethacrynic acid (EA) failed to show any significant effects on the cytotoxicity of these AAs. However, EA did potentiate the cytotoxicity of melphalan when given in combination with BSO, an effect that may be due to a more complete depletion of cellular GSH levels by the combined modulator treatment. Following a 1-hr exposure to cytotoxic-equivalent concentrations of these AAs, GSH levels decreased substantially in the case of 4HC and BCNU, but increased by 30–50% in the case of cisplatin and melphalan. BSO pretreatment largely blocked this effect of cisplatin and melphalan on cellular GSH, while it further enhanced the GSH-depleting activity of both 4HC and BCNU. On the basis of these results, it is concluded that (a) GSH affects the cytotoxicity of different AAs to different extents, (b) basal GST expression in MCF-7 cells does not play a major role in AA metabolism, (c) EA can potentiate the enhancing effect of BSO on melphalan cytotoxicity in MCF-7 cells, and (d) depletion of cellular GSH by pretreatment with BCNU or cyclophosphamide may correspond to a useful strategy for enhancing the anti-tumor activity of other AAs given in a sequential combination.

Key words: alkylating agents; drug resistance; drug sensitivity; reduced glutathione; glutathione *S*-transferase; MCF-7 cells

Alkylating agents (AAs)†, the oldest and perhaps the most important class of anti-cancer drugs, play a major role in the therapeutic treatment of both early and advanced breast cancer. Because of their characteristic steep dose–response curves, AAs are particularly well suited for use in autologous bone marrow transplantation and, as such, these drugs have been key elements of high-dose therapy protocols using bone marrow rescue [1]. The introduction of cisplatin, a non-classical alkylating agent, has further extended the efficacy of this class of drugs in the clinic [2].

It is generally accepted that tumor cell DNA is the critical target for alkylation by this class of cytotoxic drugs. The interaction between DNA and AAs is dependent, however, on a number of variables including the localized concentration of protective cellular nucleophiles, the mechanism of alkylation, and the chemical reactivity of the particular alkylating agent [3]. One important intracellular nucleophile is GSH, which can conjugate AAs and thereby prevent their reaction with DNA. Conjugation of GSH with AAs may proceed by non-enzymatic pathways and also by enzymatic pathways catalyzed by GSTs (EC 2.5.1.18), which comprise a superfamily of drug-metabolizing enzymes. GSTs can be grouped into at least five classes based on their biochemical, immunological, and structural properties (GST classes alpha, mu, pi, theta and microsomal) [4–6]. Elevated cellular GSH levels and increases in GST expression are frequently associated with resistance of tumor cells to AAs [7–10]. These

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† Abbreviations: AAs, alkylating agents, including the platinating agent cisplatin; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; 4HC, 4-hydroperoxy-cyclophosphamide; melphalan, L-phenylalanine mustard; GSH, reduced glutathione; GST, glutathione *S*-transferase; BSO, buthionine-D,L-sulfoximine; EA, ethacrynic acid; CDNB, 1-chloro-2,4-dinitrobenzene; cisplatin, *cis*-diammine-dichloroplatinum(II); IC_{90}, 90% inhibitory concentration; and DMF, dose-modifying factor, determined by dividing the IC_{90} for a given AA by the IC_{90} for the AA given in combination with one or more modulating agents, e.g. BSO and EA.

and other findings, including the observation that depletion of cellular GSH by the GSH synthesis inhibitor BSO can, in some cases, partially reverse drug resistance [7, 11–13], lend support to the hypothesis that the GSH/GST system plays an important role in acquired resistance to AAs [14]. However, the true significance of GSH-dependent metabolism for acquired AA resistance is unclear, given that BSO treatment of drug-sensitive parental cell lines often leads to a similar or even greater sensitization to melphalan [15], 4HC* and cisplatin [15–17], even though the parental cells have lower GSH levels compared with the corresponding resistant cells. Moreover, while *in vitro* data support the notion that the GSH/GST system could be a determinant of AA cytotoxicity for multiple AAs [reviewed in Ref. 14], little is known about the relative impact of GSH/GST-dependent metabolism on the cytotoxicity of different classes of AAs. It is therefore of importance to evaluate the role of GSH and GST, not only in the cytotoxicity of AAs toward drug-resistant cells but also toward the corresponding drug-sensitive parental cells, as it is to directly compare the effects of GSH depletion and GST inhibition on multiple AAs within a single cell line. This type of information is essential in order to develop a better understanding of the interactions of AAs with the GSH/GST system, both in AA-sensitive tumor cells, as well as in drug-resistant cell lines. This information may, in turn, help explain the complex patterns of AA cross-resistance that frequently characterize drug-resistant tumor cells [18].

In the present study, we have evaluated the effects of GSH depletion on the sensitivity of cultured human breast cancer MCF-7 cells to each of four structurally and mechanistically distinct AAs, in order to identify the extent to which GSH-dependent metabolism impacts on the cytotoxicity of each of these electrophilic drugs. In addition, we have characterized the effects of each of the AAs on intracellular GSH levels following acute drug exposure in order to determine whether there are substantial differences between AAs with respect to GSH depletion. Finally, we have assessed the contribution that constitutively expressed GST enzymes make to the AA sensitivity of this cell line by use of the GST inhibitor ethacrynic acid (EA). Our findings led us to conclude that there are substantial differences among the AAs, both with respect to their effects on cellular GSH, and with respect to the impact of GSH levels on the expression of AA cytotoxicity.

MATERIALS AND METHODS

Chemicals and cell line. Dulbecco's modified Eagle's medium (D-MEM) powder, penicillin/streptomycin liquid and trypsin/EDTA solution were purchased from GIBCO, Grand Island, NY. Fetal bovine serum, BSO and EA were purchased from the Sigma Chemical Co., St. Louis, MO. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and 5′-sulfosalicylic acid were obtained from the Aldrich Chemical Co., Milwaukee, WI, and cisplatin solution (1 mg/mL of 0.9% NaCl) and BCNU powder (Bristol-Meyers) from the Dana-Farber Cancer Institute Pharmacy. 4HC powder was purchased from Scios Nova, Baltimore, MD. The human breast cancer cell line MCF-7, originally developed by Dr. M. Rich of the Michigan Cancer Foundation, was grown as a monolayer in D-MEM medium supplemented with antibiotics and 10% fetal bovine serum at 37°, in 95% air and 5% CO_2, and was passaged weekly.

Cytotoxicity assays. Drugs used for cytotoxicity assays were prepared fresh before each experiment. BCNU and melphalan were dissolved in acidified ethanol (absolute ethanol containing 5% 1 N HCl) at stock concentrations of 25 and 5 mM, respectively. These stock solutions were then diluted with the same acidified ethanol vehicle to various concentrations, and 50-μL aliquots were then added to tissue culture flasks containing 5 mL of culture medium. Cisplatin stock solution was diluted with sterile 0.9% NaCl, and then 150-μL portions were added to 5 mL of culture medium. 4HC (10 mM) and BSO (5 mM) were dissolved directly in culture medium to give the indicated stock concentrations. EA was first resuspended in 1 vol. of absolute ethanol, followed by the addition of 4 vol. of medium to give a stock concentration of 5 mM. 4HC, BSO and EA were each added to cell cultures at 1:100 dilutions, as in the case of BCNU and melphalan. Equivalent values of drug vehicles were added to all samples within a given experiment. Cells in exponential growth (2 days after seeding), with or without pretreatment with 50 μM BSO (24 hr) and/or EA (15 min), were treated with various concentrations of AAs for 1 hr in the continued presence of BSO and/or EA, as indicated for each experiment.

Following a 1-hr incubation with AAs, cells were washed with PBS and harvested by treatment with 0.25% trypsin/0.1% EDTA, followed by centrifugation. Cells were then plated in duplicate at 200, 400, and 2000 per well (3 mL) in six-well plates (Falcon No. 3046) to assay for colony formation. After a growth period of 12 ± 1 days, colonies were visualized by staining with crystal violet, and those consisting of more than approximately 50 cells were counted. Results are expressed as surviving fraction, i.e. the ratio of colonies in the drug-treated as compared with the vehicle-treated control groups. Plating efficiencies of 20–50% were typically obtained.

GSH assay. GSH measurements were carried out using aliquots of cell treated exactly under the conditions of the cytotoxicity assays, with BSO and/or EA treatment as given in the legend of Fig. 1. All compounds were prepared as for the cytotoxicity assays, except that higher stock concentrations were employed (BCNU, 107 mM; melphalan, 10.1 mM; 4HC 16.7 mM). For studies on the effects of EA and AAs on cellular GSH levels, cells were treated with these compounds under the same conditions as in the cytotoxicity assay and then prepared for assay immediately after drug exposure. Total cellular GSH content [GSH + oxidized GSH, i.e. GSSG] was determined spectrophotometrically by an enzymatic

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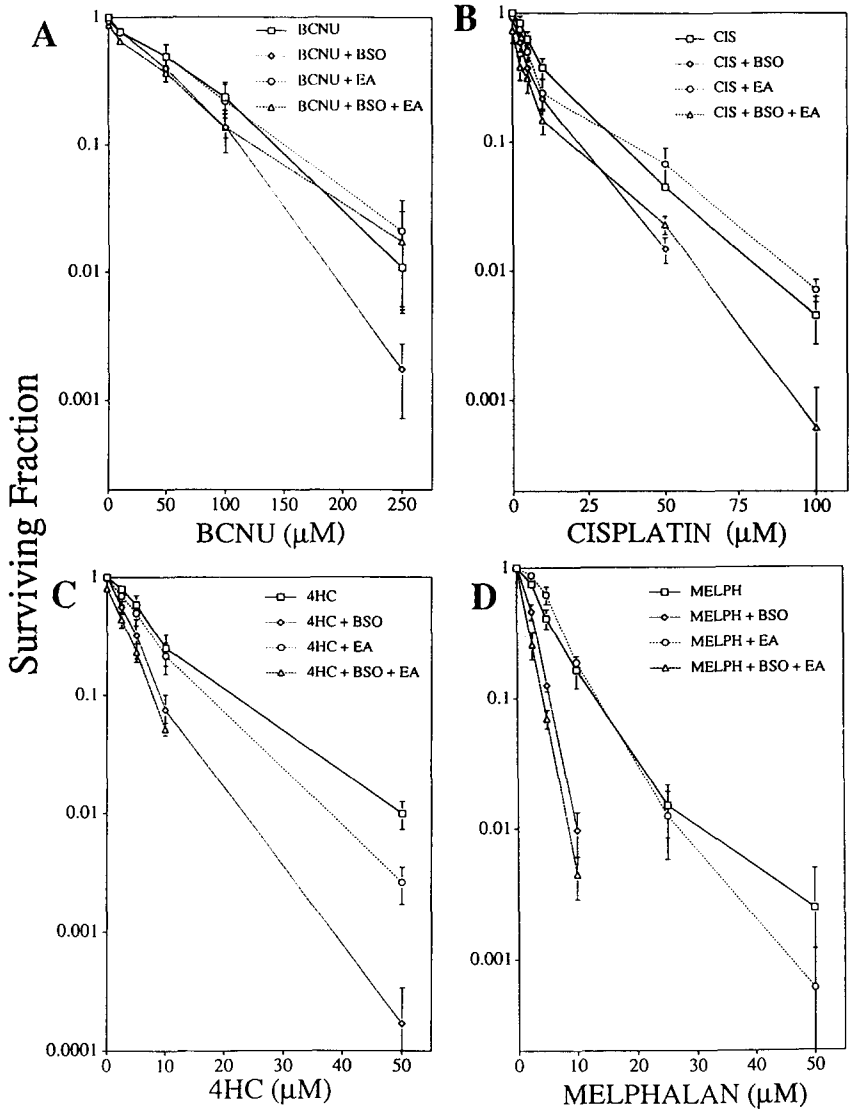

Fig. 1. Modulation of AA cytotoxicity toward MCF-7 cells by BSO, EA and BSO + EA in combination. Exponentially growing cells were treated with 50 μM BSO for 24 hr and/or 50 μM EA for 15 min, followed by coincubation with the indicated concentrations of AAs for 1 hr. Cells were plated in duplicate at each of three densities, as described under Materials and Methods, and colonies of more than 50 cells were counted ~12 days later. Results are presented as surviving fraction (mean ± SEM for at least three independent experiments) relative to drug-free controls. Data are shown for cells treated with BCNU (panel A), cisplatin (panel B), 4HC (panel C) or melphalan (panel D), as indicated.

recycling method [19]. Briefly, cells were collected by trypsinization and washed once with cold PBS at 208 *g* for 10 min at 4°. Cell pellets were resuspended in 9 vol. of ice-cold double-distilled water at a concentration of ~2×10^6 cells/mL with vigorous shaking, to which 1 vol. of 30% 5-sulfosalicylic acid was added to give a final concentration of 3%. The mixture was then incubated on ice for 10 min, followed by centrifugation (1876 *g* for 10 min at 4°). The supernatant was then assayed for GSH content.

GST activity and protein assays. Cells were untreated or were treated with EA and then collected as described above. Cell suspensions in 0.1 M sodium phosphate buffer, pH 6.5, were sonicated with a Fisher Sonic Dismembrator (model 300) for 2×10 sec on ice at an instrument setting of 35% and then were centrifuged at 11,750 *g* in an Eppendorf microcentrifuge at 4°. GST activity was determined according to Habig *et al.* [20] using 1 mM CDNB as substrate and 1 mM GSH as a co-substrate in a 1-mL reaction mixture containing 100–400 μg protein in 0.1 M phosphate buffer (pH 6.5) at 30°. In other experiments, the inherent inhibitory effect of EA on the catalytic activity of MCF-7 cytosolic GST was examined by the addition of EA directly

Table 1. Combination cytotoxicity of alkylating agents (AAs) with BSO and/or EA in MCF-7 cells

Treatment*	IC_{90}† (μM)	DMF‡
BCNU	137 ± 26	
+BSO	108 ± 16	1.27
+EA	148 ± 52	0.93
+BSO + EA	121 ± 51	1.13
Cisplatin	34.1 ± 1.8	
+BSO	20.8 ± 1.0§	1.64
+EA	32.7 ± 15.5	1.04
+BSO + EA	17.0 ± 8.5§	2.00
4HC	19.9 ± 5.0	
+BSO	9.0 ± 2.3§	2.21
+EA	15.9 ± 3.8	1.25
+BSO + EA	7.6 ± 0.8§	2.61
Melphalan	12.6 ± 3.3	
+BSO	5.3 ± 0.5§	2.39
+EA	13.4 ± 0.6	0.94
+BSO + EA	3.8 ± 0.6§‖	3.32

* Cells in exponential phase were pretreated with or without 50 μM BSO for 24 hr and/or 50 μM EA for 15 min. Cells were then exposed to one of the four AAs for 1 hr in the continued presence of BSO and/or EA, as indicated.

† Values (means ± SD) were based on full survival curves determined in 4 independent experiments for the BCNU treatment groups and 3 experiments for the other AAs.

‡ DMF (dose-modifying factors) were calculated by dividing IC_{90} values for each AA alone by the IC_{90} values for the AA in combination with BSO and/or EA. DMF values that were statistically different ($P < 0.05$) are underlined.

§ $P < 0.05$ vs AA treatment alone.

‖ $P < 0.05$ vs melphalan + BSO.

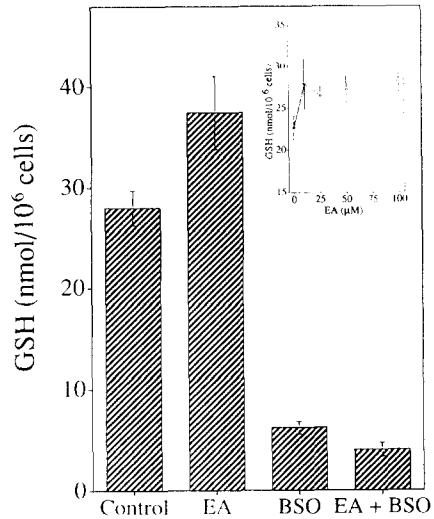

Fig. 2. Effects of BSO and EA on cellular GSH levels in cultured MCF-7 cells. Cells were treated with BSO and/or EA, using the protocol described in Fig. 1. Results are presented as mean values ± SEM for N = 6 independent experiments. *Inset:* Cells in exponential phase were treated with the indicated concentrations of EA for 1 hr, followed by preparation for GSH assay. Data points are means ± SEM for N = 3 experiments.

to cell extracts. Protein content was measured by the method of Bradford.

Data analysis. The IC_{90} values were determined from full survival curves and are based on N = 3–4 independent experiments. The IC_{90} values from one treatment group were compared with those of the next, and their statistical significance was judged by Student's *t*-test, with P values < 0.05 considered to be significant. Additionally, DMF values were calculated by dividing IC_{90} values for cells treated with AAs in the absence of BSO and/or EA by IC_{90} values for cells treated with AAs in the presence of these modulators.

RESULTS

Modulation of AA cytotoxicity toward MCF-7 cells by BSO and EA. MCF-7 cells were pretreated with the GSH synthesis inhibitor BSO (50 μM) for 24 hr to reduce cellular GSH levels. The effect of this GSH depletion on the AA sensitivity of the cell was then determined. As shown in Fig. 1 and summarized in Table 1, BSO increased the sensitivity of the MCF-7 cells to a different extent for each drug. DMF values of 1.27, 1.64, 2.21 and 2.39 were observed for BCNU, cisplatin, 4HC, and melphalan, respectively. Thus, BSO is most effective in sensitizing the cells to melphalan and 4HC and less effective in the case of cisplatin. The small enhancing effect of BSO on the BCNU sensitivity of MCF-7 cells was not statistically significant.

Since the conjugation of GSH with AAs can be catalyzed by GSTs [14], we next examined whether increases in cytotoxicity could be obtained by pretreating the cultured cells with the GST inhibitor EA. EA inhibits GSTs belonging to several classes [21–24] and has been shown to sensitize a number of tumor cell lines to alkylating agents [25–29]. However, exposure of MCF-7 cells to EA (50 μM EA for 15 min), followed by co-treatment with AAs for an additional 1 hr, failed to show any significant effects on the cytotoxicity of melphalan, cisplatin or BCNU. In the case of 4HC, a small increase in cytotoxicity was observed with EA treatment, but this did not reach statistical significance (DMF = 1.25; $P > 0.05$) (Table 1). As for the three-drug combinations, i.e. AA + BSO + EA, only in the case of melphalan + BSO was a significant further increase in cytotoxicity obtained upon addition of EA ($P < 0.05$ vs melphalan + BSO alone) (Table 1). In control experiments, neither BSO nor EA alone exhibited significant toxicity, with survival fractions of >0.9 compared with the controls obtained in all experiments. Moreover, BSO + EA in the absence of AA gave a survival fraction of 0.85 ± 0.11 (N = 13).

Effects of BSO and EA on cellular GSH content. We next examined the effects of BSO and EA on cellular GSH levels. As shown in Fig. 2, treatment of MCF-7 cells with 50 μM BSO for 24 hr decreased cellular GSH by about 80% (6.2 vs 28.0 nmol GSH/10^6 cells). Addition of EA (15-min exposure to 50 μM EA) to BSO-treated cells further reduced

Table 2. Effect of EA treatment on GST activity

Experiment A: Cell-free extracts*		Experiment B: Intact cells	
EA (μM)	Relative GST activity† (% of control)	EA‡ (time)	Relative GST activity§ (% of control)
0	100	0	100
5	70.4 ± 12.9	15 min	91.6 ± 6.2
10	57.0 ± 9.8	75 min	83.1 ± 5.8
50	7.5 ± 7.2		

* GST activity was measured by using CDNB as substrate in the presence of various concentrations of EA.

† GST activity in the 0 μM EA group was 2.6 ± 0.4 nmol CDNB conjugated/min/mg (mean ± SD, N = 3).

‡ Cells were treated with 50 μM EA for 15 or 75 min, as indicated.

§ GST activity in control cells was 2.8 ± 1.2 nmol/min/mg (mean ± SD, N = 4).

cellular GSH by about 35% (4.0 vs 6.2 nmol/10^6 cells, $P < 0.05$). Unexpectedly, EA treatment in the absence of BSO *increased* intracellular GSH by 30% ($P < 0.05$). This small elevation of cellular GSH occurred as early as 5 min after the addition of EA to the cells (data not shown) and was also observed in longer incubations (1-hr EA treatment) and at concentrations of EA varying from 12.5 to 100 μM (Fig. 2, inset).

Inhibition of GST activity by EA. The inhibitory effect of EA on GST activity in MCF-7 cells was determined in studies carried out in cell-free extracts. MCF-7 GST activity assayed with the general GST substrate CDNB was inhibited ~40% by 10 μM EA and >90% at 50 μM EA. A similar degree of GST inhibition has been reported in EA-treated human tumor cells [27]. A large fraction of this inhibition appears to be reversible, since only ~20% inhibition of GST was obtained at 50 μM EA when the inhibitor was removed by washing EA-treated MCF-7 cells with drug-free phosphate-buffered saline prior to *in vitro* analysis of GST activity (Table 2B).

Effects of AAs on cellular GSH levels. As shown above, BSO depletion of cellular GSH increased the sensitivity of MCF-7 cells to three of the four AAs, albeit to a different extent for each one. Since the AAs that are chemosensitized most effectively by GSH depletion could be those that are extensively inactivated via GSH conjugation, a process that could itself lead to GSH depletion, we next examined the effects of the four AAs on cellular GSH. GSH levels were compared in MCF-7 cells exposed to each of the AAs at cytosolic equivalent concentrations, with or without pretreatment with 50 μM BSO. As shown in Fig. 3, GSH was increased by 30–50% after treatment of MCF-7 cells for 1 hr with either cisplatin or melphalan at concentrations ranging from the IC_{90} to 4 times the IC_{90}. By contrast, intracellular GSH decreased dramatically with increasing concentrations of either 4HC or BCNU. The decrease of intracellular GSH was more substantial in cells treated with BCNU than 4HC at cytotoxic-equivalent concentrations; for example, at $4 \times IC_{90}$, GSH decreased to 35% of control following BCNU treatment but only to 66% of control in 4HC-treated cells. However, at $8 \times IC_{90}$, the extent of GSH depletion was similar with both drugs. The increase of cellular GSH after exposure to cisplatin or melphalan was partially blocked in cells pretreated with BSO, while the extent to which 4HC and BCNU depleted cellular GSH was further enhanced when the cells were pretreated with BSO (Fig. 3).

DISCUSSION

The present study establishes that cellular GSH depletion differentially impacts the cytotoxicity of each of four commonly used AAs. BSO pretreatment of MCF-7 cells sensitized the cells toward melphalan and 4HC to the greatest extent, followed by cisplatin. By contrast, cellular GSH levels were found to be less important for BCNU cytotoxicity toward MCF-7 cells. Our finding that the cytotoxicity of melphalan and 4HC can be enhanced by GSH depletion is consistent with earlier *in vitro* and *in vivo* studies [11, 12, 30]. Mechanistically, the effects of GSH depletion on the cytotoxicity of these two AAs could involve drug inactivation, e.g. by conjugation of GSH to melphalan or to the 4HC derivative phosphoramide mustard, or perhaps to their corresponding aziridinium derivatives. In the case of melphalan, drug inactivation via the formation of AA–GSH conjugates can proceed spontaneously, or may be GST-catalyzed [31]. Alternatively, the observed effect of GSH levels on melphalan and 4HC cytotoxicity could involve the quenching by GSH of DNA-monoadducts formed as intermediates during the cross-linking process, by analogy to the quenching by GSH of cisplatin- and BCNU-derived DNA-monoadducts [32, 33]. Further support for the importance of cellular GSH as a determinant of the sensitivity of tumor cells to 4HC comes from studies of both human and animal tumor cells made resistant to 4HC, where one of the factors contributing to resistance appears to be the elevation of intracellular GSH [34, 35]. By contrast, in the case of cisplatin, although in some cell lines resistance development is associated with increased GSH [13, 36–38], this phenomenon is not universal [39–41]. This discrepancy may be due to the complexity of factors involved in cisplatin resistance [42], and to the intrinsic biology of the tumor type and the cell lines

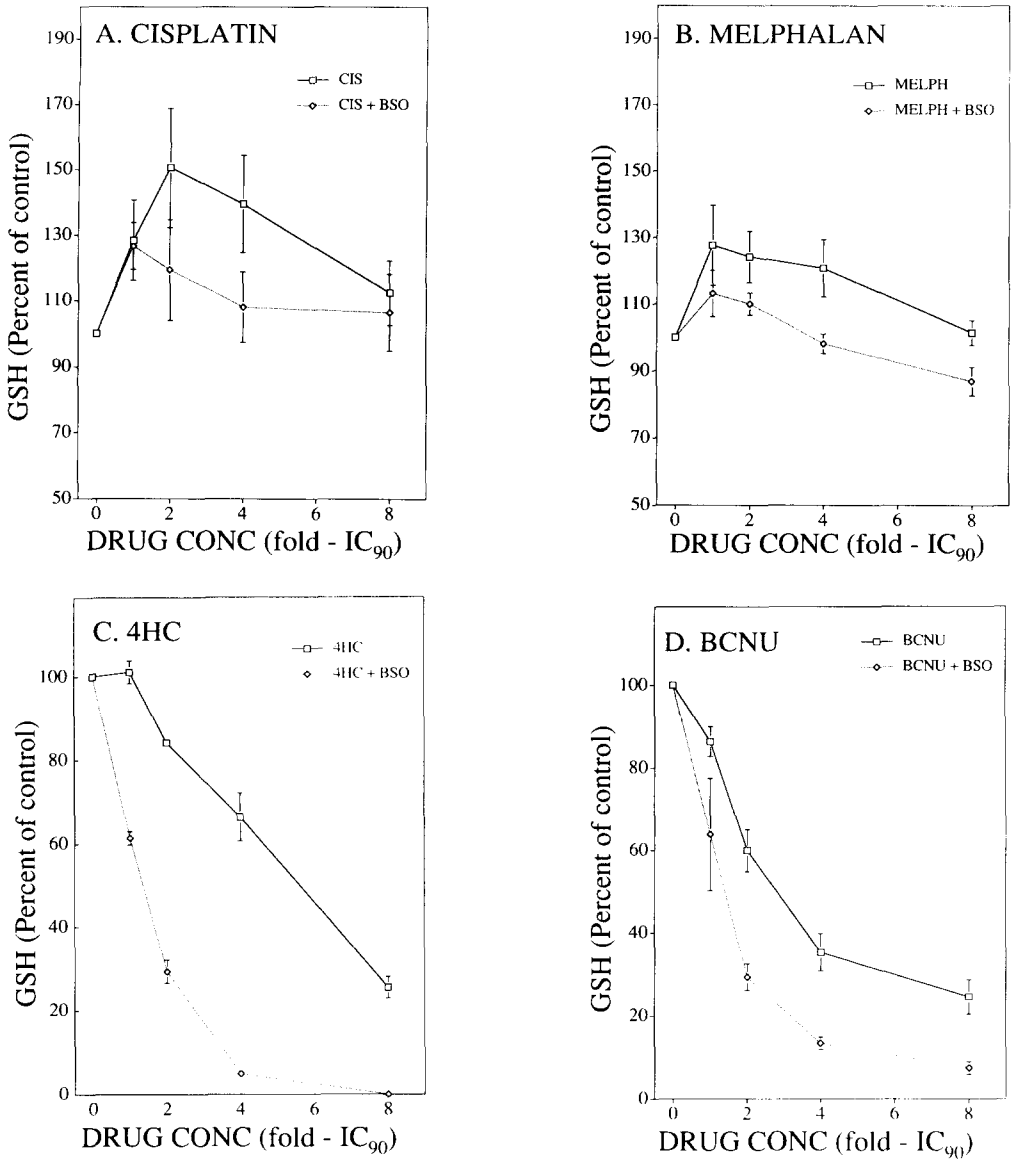

Fig. 3. Effects of AA treatment on GSH levels in MCF-7 cells. Cells in exponential phase were treated with or without BSO (50 μM for 24 hr), followed by the four AAs at the indicated concentrations for 1 hr. Cells were then trypsinized, and GSH levels were determined, as described in Materials and Methods. Results shown are mean values ± SEM for N = 4 experiments for cisplatin and melphalan, and N = 3 experiments for 4HC and BCNU, and are expressed relative to the GSH levels measured prior to AA treatment. The corresponding 100% values for GSH, expressed as nmol GSH/10^6 cells, were 26.3 ± 1.4; 27.6 ± 1.9, 23.4 ± 0.9 and 26.9 ± 2.1 for the untreated controls, and 6.8 ± 0.7, 6.2 ± 0.6, 4.2 ± 0.9 and 2.9 ± 0.5 for the BSO-treated controls, for the experiments using cisplatin, melphalan, 4HC and BCNU, respectively.

used in the investigations [43, 44]. Similar to our results in MCF-7 cells, only a minimal potentiation of BCNU cytotoxicity by BSO has been observed in rat brain tumor cells [45] and in human melanoma cells [46]. These data, together with the direct comparative experiments of the present study, indicate that BSO may be best suited for further clinical study as a chemosensitizer for melphalan and 4HC.

Previous studies using EA or other GST inhibitors have provided good evidence for the importance of GSTs as a determinant of tumor cell drug sensitivity for AAs such as melphalan and chlorambucil [9, 10, 25, 27], a finding that is consistent with the catalytic activity exhibited by several individual GST enzymes with these drug substrates [14, 22, 31]. In the present study, however, treatment of MCF-7 cells with EA had no significant effect on the

cytotoxicity of any of the AAs examined, despite the fact that EA significantly inhibited MCF-7 cell GST activity when assayed in a cell-free system. One possible explanation for this finding is that the specific GST isoenzymes expressed in MCF-7 cells may not be active in AA metabolism. Alternatively, since MCF-7 cells have a very low endogenous GST activity (~3 nmol CDNB conjugated/min/mg; Table 2), non-enzymatic conjugation of GSH to AAs may predominate such that GST inhibition has little net effect on AA cytotoxicity. Although EA only partially inhibited MCF-7 GST activity when assayed in an intact cellular system following removal of unbound inhibitor (Table 2B), EA inhibition of GST is known to be reversible [21] such that the partial inhibition that we observed in these experiments likely reflects the irreversible fraction of GST inactivation. In agreement with this possibility, EA has been found to enhance the cytotoxicity of some anti-cancer drugs at doses that only slightly inhibit GST activity in tumor cells [25, 27].

Although EA had no effect of its own on the cytotoxicity of melphalan toward MCF-7 cells, it potentiated the cytotoxicity of melphalan given in combination with BSO. This suggests that BSO and EA act independently in the expression of melphalan cytotoxicity, a finding that may have potential clinical application. Synergy or potentiation between BSO and EA has been reported recently in a chlorambucil-resistant mouse fibroblast cell line [10] and in Adriamycin®-resistant mouse leukemia cells that show cross-resistance to mitomycin C [47]. However, the underlying mechanisms for these effects of EA may be quite different. EA treatment resulted in a *decrease* of GSH in these two lines [25, 28], whereas in our study EA was found to *increase* intracellular GSH. An elevation of cellular GSH following EA treatment has been observed in other tumor cell lines as well [29, 48]. Moreover, a corresponding change occurs in K562 leukemia cells treated with another GST inhibitor, bromosulfophthalein [48]. Since EA and bromosulfophthalein are not only GST inhibitors but also serve as GST substrates [49], these agents may consume cellular GSH via GST-catalyzed conjugation reactions. This could lead to a rebound in cellular GSH synthesis and thereby account for the modest increases in intracellular GSH content that we have observed following EA treatment. This hypothesis is strengthened by our observation that despite its stimulatory effect on cellular GSH levels, EA further depleted GSH when given to BSO-pretreated cells, where GSH resynthesis is blocked. This raises the interesting possibility that the further sensitization of MCF-7 cells to melphalan by EA in combination with BSO (Table 1) could result from the more complete depletion of GSH that occurs in the EA-treated cells, rather than from the inhibition of GST *per se*.

Treatment of MCF-7 cells with AAs, at cytotoxic-equivalent concentrations was found to lead to either increases or decreases in cellular GSH, depending on the AA. The 30–50% elevation of GSH that we observed following cell exposure to moderate concentrations of cisplatin and melphalan may reflect an adaptive response to these electrophilic drugs, while the reversal of this increase seen at high drug concentrations could be due to GSH consumption resulting from AA conjugation. The blocking of this increase in BSO-pretreated cells suggests that this adaptive response involves an increase in GSH synthesis. In the case of 4HC and BCNU, the substantial decrease of cellular GSH after a 1-hr drug exposure suggests that the overall rate of GSH consumption under these conditions exceeds the rate of GSH recovery. However, the mechanisms involved in GSH depletion by 4HC and BCNU are likely to be different: reduction of GSH by 4HC likely occurs through conjugation of GSH with several of its metabolites [50] and can even be used to predict tumor sensitivity to this compound [51], while BCNU decreases GSH primarily by a mechanism involving inhibition of GSH reductase [52], an enzyme in the GSH-redox cycle that is required for maintenance of normal reduced GSH levels [53]. In agreement with this idea, the depletion of GSH by 4HC in our studies is linear with drug concentration and proceeds to near completion, whereas the effects of BCNU on GSH appear to be saturable and do not result in complete GSH depletion, even in BSO-treated cells (Fig. 3). The more effective GSH depletion conferred by 4HC, and to a lesser extent BCNU, when combined with BSO may relate to the fact that these AAs consume a fixed amount of GSH, such that the *percent* GSH depletion is higher in BSO-treated cells. Independent of the precise mechanisms involved, our findings indicate that the combination of 4HC with BSO provides a useful way to achieve a near-complete depletion of cellular GSH for experimental studies (Fig. 3).

These findings also provide an experimental basis for testing novel combinations of AAs in the treatment of breast cancer. One possibility that has not been explored thus far in the clinic [54] could be based on the use of 4HC or BCNU to first deplete GSH and thereby sensitize the tumor to subsequent treatment with melphalan, cisplatin or 4HC. Our findings in the present study may, in part, explain the effectiveness of an experimental protocol involving 4HC followed by cisplatin for treatment of leukemic cells [55]. This concept could be extended to derivatives of either 4HC or BCNU, such as deschloro-4-hydroperoxy-cyclophosphamide, a congener of 4HC [50], and *N*,*N*′-bis(*trans*-4-hexyl)-*N*′-nitrosourea (BHCNU) [56, 57], which alone have no or low cytotoxicity but are, respectively, able to conjugate GSH or inhibit GSH reductase [50, 56, 57].

In summary, the present study establishes (a) in MCF-7 cells, cellular GSH but not GST is an important determinant for the cytotoxicity of melphalan and 4HC, and to a lesser extent cisplatin and BCNU; (b) EA and BSO can synergistically enhance melphalan anti-tumor activity in MCF-7 cells, an observation that may have some clinical significance; (c) the extent to which individual AAs deplete cellular GSH does not correlate with the sensitivity of the tumor cells to the AA; and (d) the sequential combination of either 4HC or BCNU followed by other AAs may correspond to a synergistic combination with high anti-tumor activity, a possibility that requires further investigation.

Acknowledgements—The authors wish to thank Dr. Emil Frei III for his support and encouragement during the course of these studies. This work was supported in part by Grant CH-487 from the American Cancer Society (to D.J.W.).

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